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The Swelling Factor in Cellulose Hydrolysis

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THE early changes occurring during the degradation of cotton in the field have been studied by Marsh and coworkers [6]. It was found that attack by an organism leads to a rapid increase in pH, to changes in the cotton which are responsible for its increased adsorption of Congo red, and to increased swelling of the cotton in alkali. The action of cellulolytic filtrates on cotton sliver is similar to that of the organism in that swelling in 18% alkali is greatly increased [5]. This change occurs before any reducing sugar, loss in tensile strength, or change in degree of polymerization is detectable. Treatment of the fiber for as little as 2 hrs. at 30°C has given marked increases in swollen weight even after dilution of filtrates by 1 : 100. Even action on carboxymethyl cellulose may not be detectable under such mild conditions. Marsh showed that the factor responsible is destroyed by heat, is adaptive, and is presumably enzymatic. He has called it "*S* factor," the *S* referring to the swelling effect.

Modifying cotton fiber in a highly specific manner by enzymic action is interesting to observe. Whether the changes effected have any practical application is unknown. The specificity of the action depends upon the purity of the *S* factor (*i.e.*, freedom from other enzymes) and the nature of the action of *S* factor on cotton fiber.

The primary object of the present paper is to examine the relationship of the *S* factor to other cellulolytic factors, and to attempt to determine the role of the *S* factor in the pattern of enzymes involved in cellulose breakdown. Most of the results on *S* factor are in agreement with the findings of Marsh [3, 5].

Methods

Measurement of S *Factor* (SF) *Activity*

A 250-mg. sample of cotton sliver is incubated with 15 ml. of enzyme solution for 2 hrs. at pH 5.2 (*M*/20 citrate) and 30°C (unless otherwise stated). The sample is removed, partially dried between blotters, placed in 50 ml. of 18% NaOH, and shaken for 15 min. at 30°C. The swollen sample is collected in a Gooch crucible, transferred to a fritted glass crucible, and centrifuged (at about 550 g.) for 10 min. to remove excess alkali. The sample is finally transferred to a weighing bottle and weighed. This is essentially the procedure developed by Marsh [4].

Our results are expressed as the weight of the swollen test sample minus the weight of the swollen control (*i.e.*, no enzyme treatment). The maximum difference is about 250 mg. under the conditions of our test. Since the curve of increase in weight *vs.* enzyme concentration is not a straight line at high enzyme concentrations, a unit has been defined as the amount of enzyme required to obtain an increase in swelling of 50 mg. beyond the control value.

Measurement of Cx *Activity*

Cx activity is measured by the reducing sugars (as glucose) produced by the action of the enzyme on 10 ml. of 0.5% carboxymethyl cellulose (*CMC*) of low viscosity and of low degree of substitution (Hercules *CMC* 50T, D.S. = 0.52) in *M*/20 citrate buffer at pH 5.4 and 30°C. The *Cx* unit used here is the amount of enzyme which under the above conditions gives a reducing value of 0.40 mg. reducing sugar per ml. of reaction mixture in 2 hrs. It differs from the regular *Cx* unit [11] in that the time and temperature have been changed to conform with the conditions of the *S* factor assay.

Preparation of Cellulolytic Filtrates

The cellulolytic filtrates are prepared by growing the organism on cellulose in shake flasks [12]. At the end of the incubation period the culture fluid is filtered through medium-porosity glass filters. The clear solutions obtained are kept in a refrigerator. Merthiolate (0.01%) is usually employed as a preservative.

Results

Enzymatic Nature of S *Factor*

We have confirmed the observations of Marsh that the *S* factor can be destroyed by heat (autoclave

121°C, 15 min.), and that it is adaptive in most organisms. *S* factor will not pass through cellophane membranes, nor is its activity diminished on dialysis. It can be precipitated from solution by two volumes of alcohol or acetone. The pH-activity curves and the temperature-activity curves (see later sections) are of the type generally associated with enzymatic reactions. The pH stability is greatest near pH 5.0, and least below pH 3.0 and above pH 9.0.

Points of Similarity Between Cx *and* S *Factor*

Adaptive nature.—Both enzymes are adaptive in most cellulolytic microorganisms, a great increase in potency being observed in the presence of cellulose as substratum [4, 11]. The only exception observed is *Aspergillus luchuensis* QM 873, in which *Cx* is found to be produced on noncellulosic, as well as on cellulosic, substances. Re-examination of this organism reveals that the *S* factor is also constitutive in this fungus.

In noncellulolytic organisms growing on sugars, we have been able to detect neither factor, except possibly in *Rhizopus nigricans,* where a trace of *S* factor is evident on long incubation.

Simultaneous occurrence of enzymes.—In a series of commercial preparations of enzymes, *Cx* and *S* factor were either both present or both absent. They were present in cellulase, (Takamine); pectinol 10M and 19AP (Rohm & Haas); and lipase (General Biochemical Co.). They were absent under the test conditions (50°C, 2 hrs., pH 5.0) in emulsin, pepsin, ficin, Rhozyme PF, β-glucuronidase, hyaluronidase, takadiastase, and malt diastase.

Both enzymes withstand dialysis and solvent precipitation. Both are destroyed by autoclaving. At 55°C, both are relatively stable at pH 5.5, but are

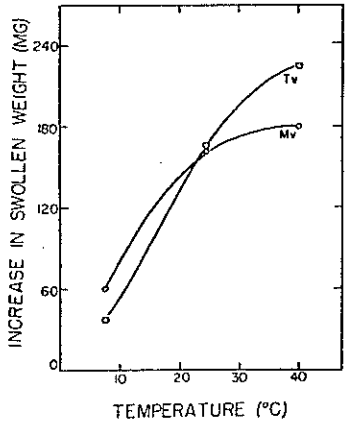

FIG. 1. *Temperature* vs. S *factor activity.* Tv = *Filtrate of* Trichoderma viride *QM 6a.* Mv = *Filtrate of* Myrothecium verrucaria *QM 460.*

TABLE I. INHIBITION OF *S* FACTOR BY CELLOBIOSE AND GLUCOSE

Filtrate	QM No.	pH of reaction mixture	Inhibition (%) Cellobiose* 1.0%	0.1%	Glucose 1%
Myrothecium verrucaria	460	5.7	73	—	22
Streptomyces sp.	B814	5.65	60	—	56
Trichoderma viride	6a	5.65	67	—	0
Trichoderma viride	6a	3.15	73	38	22
Penicillium pusillum	137g	5.65	90	69	20
Penicillium pusillum	137g	3.15	82	40	3

* Weight relationship to cotton sliver: at 1% cellobiose, the ratio sugar/cotton = 0.4; at 0.1%, the ratio = 0.04.

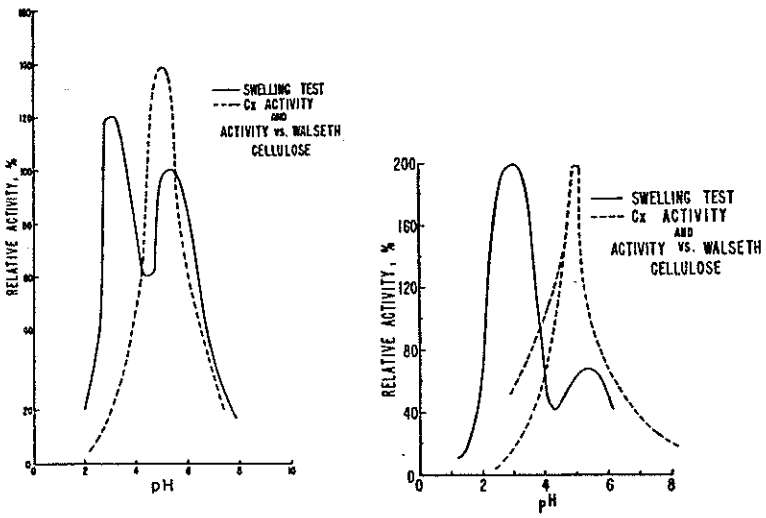

FIG. 2. *pH-activity curves of cellulolytic filtrates. These curves are based on several sets of data. The "relative activity" is based on a determination from a control dilution curve.* Left—Trichoderma viride *filtrate.* Right—Penicillium pusillum *filtrate.*

inactivated at pH 3.0 and pH 9.6. Urea (1.67*M*) inhibits both *Cx* and *S* factor.

Both enzymes have the same type of temperature-activity curves. The activities fall off at a lower temperature for *Myrothecium verrucaria* than for *Trichoderma viride* (Figure 1).

Both enzymes are inhibited by methocel and by cellobiose. *Trichoderma viride* filtrate was tested for the methocel inhibition, filtrates of this organism being greatly affected:

	Inhibition	
	Cx	*S* factor
0.1% methocel	92%	91%
0.01% methocel	81%	80%

Cellobiose inhibition of *Cx* was reported previously [10]. It was shown that in most cases cellobiose had a much greater inhibiting effect than other sugars, including glucose. There were exceptions, however, in which the degree of inhibition by 1% cellobiose was low, and equalled by glucose. The data for inhibition of *S* factor (Table I) show that in all cases but one (*Streptomyces* sp.), cellobiose inhibition is much greater than glucose inhibition. Our previous data show *Cx* activity of this organism to be inhibited much more by cellobiose than by glucose.

TABLE II. RATIO OF *S* FACTOR/*Cx* FOR FILTRATES OF SEVERAL ORGANISMS GROWN ON CELLULOSE

Name	QM No.	Activity* *Cx*	Activity* *S* factor	Ratio *SF/Cx*
Trichoderma viride	6a	3.5	100	29
Myrothecium verrucaria	460	5.0	96	19
Streptomyces sp.	B814	2.6	8.6	3.3
Chaetomium globosum	459	0.5	1.2	2.4
Penicillium pusillum	137g	16.0	21.0	1.3
Pestalotia palmarum	381	2.6	3.1	1.2
Aspergillus flavus	10e	0.9	0.64	0.7
Aspergillus niger	458	1.4	0.6	0.4

* Activity in units/ml.

Differences Between Cx *and* S *Factor*

pH-activity curves.—Marsh [6] pointed out to us an odd bimodal pH-swelling activity curve for his filtrate of *Myrothecium verrucaria*. Our filtrates of this organism do not behave in the same manner, but have a single, broad-optimum range. Filtrates of two other organisms, however, have well-defined bimodal pH-swelling curves. The optima are at pH 3.0 and at pH 5.2 in both cases (Figure 2). The two peaks are of about the same height for *Trichoderma viride*, but the acid peak is consistently higher than the second peak in *Penicillium pusillum*. These bimodal curves are quite different from the single-peak pH curves usually found for *Cx* activity. The main difference is the presence of an additional peak for *S* factor at pH 3, at which level *Cx* is not active.

Nickerson [8] made a similar observation on a different system—namely, the effect of pH on respiration of dermatophytes. He found that the dip between the peaks could also be obtained if salt concentration were plotted against activity, from which it could be deduced that the phenomenon is attributable to the effect of salt on protein. Marsh [6] reported that salts inhibit the *S* factor. In checking this, we found that in all cases swelling activity falls off rapidly with increasing salt concentration, and that a dip is discernible, giving a bimodal curve, but only at pH 4.5. (At pH 3.0, no dip is detectable; at pH 5.2, a slight plateau only is in evidence. NaCl concentration at the dip = 1.0%–1.3%; citrate concentration = 1.25%.) It is probably significant that the dip in the salt concentration curve occurs at the same pH as the dip in the pH-activity curve. We

TABLE III. CHANGES IN RATIO OF *S* FACTOR/*Cx* DURING PURIFICATION

Filtrate	Treatment	Activity† *Cx*	Activity† *SF*	Ratio *SF/Cx* (pH 5.3)
Myrothecium verrucaria	—	3.2	64	20
Myrothecium verrucaria	Solka Floc* adsorption (pH 5.0)	2.2	17	7.7
Penicillium pusillum	—	21	55	2.6
Penicillium pusillum	Solka Floc adsorption (pH 3.3)	11	5.5	0.5
Trichoderma viride	—	3.2	100	31.3
Trichoderma viride	Solka Floc adsorption (pH 4.9)	2.2	35	15.9

* Solka Floc is a finely divided wood cellulose (Brown Co., Berlin, N. H.). The values shown are for the residual solution —*i.e.*, for *unadsorbed* enzyme.

† Activity in units/ml.; pH 5.3.

have not investigated this point further. It is entirely probable that our failure to obtain a bimodal curve for *Myrothecium verrucaria* is due to the use of conditions different from those of Marsh.

Jermyn [2] reported instances of bimodal pH-activity curves for β-glucosidases in the filtrates of *Aspergillus oryzae*. He believed that such curves indicate the presence of two β-glucosidases.

Relative ratios of S *factor*/Cx.—We have been unable to obtain either *Cx* free of *S* factor or vice versa. As a result, we have found it necessary to use a less direct approach. If two factors are involved, the ratio of one activity to the other should vary with the organism producing them, with the conditions of growth of the organism, and with the various steps in purification or inactivation. In an attempt to see how widely the ratios differ, a series of 80 organisms was grown on cotton duck strips in test tubes. The range of ratios was found to be 0.4–42, the values being distributed uniformly. Since the ratios do vary (Table II), it is apparent that two or more factors are involved.

Cellulolytic enzymes are adsorbed on cellulose at low pH [11]. The data (Table III) indicate that a preferential adsorption of *S* factor takes place, since the residual solution has a lower *S* factor/*Cx* ratio than the original. Concentration of filtrates by acetone precipitation did not change the ratios for three filtrates tested. Heat inactivation at pH 3.0 had no effect on the ratio in *Trichoderma viride* filtrate, but lowered the ratio in a filtrate of *Penicillium pusillum*.

Growth of *Myrothecium verrucaria* at low pH (4.1–4.5) gave a filtrate of lower ratio ($R = 7$) than growth at higher pH levels (6.6–7.0; $R = 16$). This agrees with adsorption being greater at the low pH's and swelling factor being more readily adsorbed than *Cx*.

Movement of Cx *and of* S *factor on paper chromatograms.*—Chromatograms showing a multiplicity of *Cx* factors in filtrates have been published [9]. If *Cx* and *S* factor differ, their movement on paper might be different. Unfortunately, the assay of fragments of paper chromatograms for *S* factor activity is unsatisfactory, probably because of the great dilution of enzyme. In the swelling test, the aliquot of the strip is placed in 15 ml. of *M*/20 buffer containing the fiber, whereas in the *CMC* test, much less volume (1 ml.) is employed. The dilution factor has been partially overcome by prolonged incubation periods (19–23 hrs. at 40°C).

It was observed that in *Myrothecium verrucaria* filtrates, the *S* factor moves slowly on the chromatogram, and is associated with the first *Cx* spot. No detectable *S* factor accompanies the fast-moving *Cx* fraction.

In *Pestalotia palmarum* filtrates, there are at least three *Cx* components. The major *Cx* component moves rapidly, and is found near the solvent front (Figure 3). A second *Cx* component moves more slowly (it is not sharply defined in Figure 3 because a large sample was placed on the chromatogram), and the third component remains at the starting

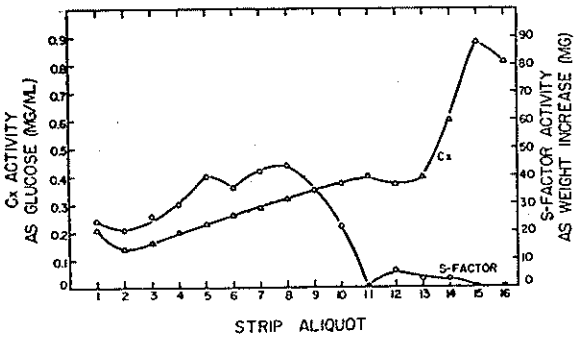

FIG. 3. *Location of various types of activity factors on paper chromatograms. Filtrate of* Pestalotia palmarum.

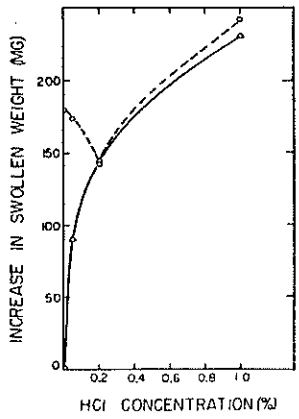

FIG. 4. *Effects of acid and enzyme on subsequent alkali swelling.* △——△, *Fiber was autoclaved with hydrochloric acid at 121°C for 30 min.; then it was washed, blotted dry, and incubated with cell-free filtrate of* Trichoderma viride *for 2 hrs. at 30°C; the swollen weight in 18% NaOH was determined.* O - - - O, *Acid hydrolysis was carried out as above; then the fiber was incubated with cell-free filtrate of* Trichoderma viride *for 2 hrs. at 30°C; the swollen weight in 18% NaOH was determined. (The fiber disintegrates on autoclaving with 1% HCl.)*

point. The *S* factors appear to be at least two in number. While the dip shown in the curve is slight, it has been found to appear with all strips tested. Neither *S* factor is related to the fast-moving *Cx* component.

Nonenzymatic Factors Affecting Swelling

In order to better understand the action of the *S* factor, other conditions affecting the swelling of cotton fibers in NaOH have been investigated.

Effect of dilute mineral acids.—When fiber is autoclaved (121°C) with as little as 0.05% HCl, there is a marked increase in subsequent alkali uptake. As the concentration of HCl is increased to 1%, the alkali uptake increases and the fiber disintegrates (Figure 4).

If acid and enzyme act on different linkages, the action of both should exceed the action of either. Treatment with 0.05% HCl followed by treatment with enzyme (Figure 4) gives swelling equal to that obtained by enzyme alone (when allowance is made for loss in weight of the acid-treated fiber in alkali). The action of both acid and enzyme seems to be on the same linkage.

Effect of alkali.—Pretreatment of fiber with alkali has little effect on subsequent swelling. A 30-min. boil with 1% NaOH gave an increase of only 27 mg. in swollen weight. Treatments which may be expected to remove pectic materials (0.1*N* NaOH; 0.5% $(NH_4)_2Ox$ at 60°C for 19 hrs.) gave no significant increases in alkali swelling.

Effect of other compounds.—Reducing agents (0.5*M* $NaHSO_3$; $Na_2S_2O_3$) had no effect on subsequent alkali swelling. Oxidizing agents varied considerably in their action. A very marked increase (16 hrs. at 30°C) was obtained by using 0.005*M* periodate (91 mg.) or permanganate (55 mg.). Under similar conditions, the amount of oxidizing agent to produce appreciable swelling was *M*/2 for hypochlorite (pH 11) and *M*/1 for hydrogen peroxide (pH 3.7). Urea (10% at the boil for 30 min.) had little, if any, swelling action.

Effect of maceration.—While cutting the sliver into small pieces with scissors does not lead to increased alkali swelling, maceration in a Waring Blendor for 5 min. results in a maximum alkali-swelling value (221 mg.). This treatment is reported to remove at least part of the primary wall [13].

Discussion

Site of Action of S Factor

The cotton fiber is surrounded by a primary wall which has a restrictive effect on swelling in alkali and on uptake of Congo red. Damage to the primary wall by various types of mechanical, chemical, or biological actions is detectable by an increase in alkali swelling or an increase in Congo red staining. The fact that mechanical tearing, as by a Waring Blendor, gives an increase in swollen weight of the same magnitude as that obtained by enzyme or acid is an indication that the primary site of action is probably the same in all cases. The rapidity with which enzyme action takes place supports the view that the action is on the primary wall.

An analysis of the primary wall, removed by treatment in a Waring Blendor [13], gives 54% cellulose, 14% protein, 9% pectic substance, 8% wax, 3% ash, and 4% cutin (?). Might *S* factor be active on one of the noncellulosic components? The cotton sliver used in our experiments was treated to remove the waxy materials. The action of *S* factor appears to be unaffected by the presence of the waxy constituents. We agree with Marsh that it seems odd that wax does not interfere with the reaction. The large protein component is a possible site, but action of proteases and of boiling NaOH does not increase the alkali swelling. Nor is the pectic substance the substrate for *S* factor, since the reagents used to remove pectic materials did not increase swelling. Indeed, many of the filtrates most active in *S* factor contain no detectable pectinase (unpublished data). Removal of any of these materials (wax, pectin, protein) does not increase the swelling of fibers in alkali, nor does it affect the action of *S* factor on swelling. While these data indicate that *S* factor does not act on noncellulosic components, they are by no means exhaustive. Nothing is known of the possible linkages existing between the components, or of agents active in splitting those linkages. The fact remains, however, that the netlike arrangement of cellulose fibrils is the only known *structure* in the primary wall, and, as such, may be capable of restricting swelling. The other components are imbedded between the microfibrils.

A further observation indicates that the action of *S* factor is on cellulose. *S* factor is an adaptive enzyme. It is produced only when the organism is grown on cellulose. The cellulose does not have to

be cotton cellulose containing the pectin, protein, and wax of the primary wall. Any source of cellulose will suffice. We consider that the linkage on which *S* factor is operative is a common linkage in cellulose, and postulate that the action of *S* factor is not limited to the cellulose of the primary wall. Its action on that cellulose, however, is readily detectable by the swelling technique used in this work.

The effect of periodate at pH 4–5 is in agreement with the theory that increased alkali swelling is due to a rupture of cellulose chains. Periodate acts on cellulose to form the dialdehyde. "The dialdehyde formed under these conditions is no more susceptible to acid hydrolysis than are normal glucosidic linkages of cellulose. They are, however, very sensitive to alkaline cleavage" [7]. As with the enzyme effect, the action of periodate can be very rapid, and thus presumably be limited to the primary wall. Periodate splitting of cellulose requires a later alkali step. While proof is lacking, we believe that the enzyme splitting is complete before the introduction of the fiber into the alkali.

The early action of cellulolytic filtrates on cotton fiber was recently investigated by Blum and Stahl [1]. A loss in tensile strength of 30% occurred within 3 days, but no further loss took place over a period of 60 days. No changes could be detected in degree of polymerization, in loss in weight, or in crystallinity. It should be pointed out that the effect on swelling in alkali is a much more rapid and sensitive measure of activity. Filtrates of *Myrothecium verrucaria* of the type used by Blum and Stahl have a marked effect on the swelling capacity within 1 hr., even after a tenfold dilution. The loss in tensile strength was attributed by the above authors to a combination of surface action and internal degradation of a highly specific type—*i.e.*, along spiral planes.

Place of S *Factor in Cellulose Hydrolysis*

In our consideration of the mechanism of cellulose breakdown by cellulolytic filtrates, we began with the assumption that each organism produces a single enzyme of each type—*i.e.*, one C_1 acting on native cellulose to liberate chains of anhydroglucose units, one *Cx* hydrolyzing these chains, and one cellobiase. Of these, C_1 and *Cx* act extracellularly, and cellobiase usually acts within the cell. Our work on *S* factor also began under these assumptions. This explains our early attempts to determine whether *S* factor was identical with or different from *Cx*. Now there appear to be several *Cx*'s in a filtrate from cultures of a single organism [2, 9] differing in rate of movement in paper-strip chromatography. Enzyme fractions can also be obtained from various types of column which differ in their movement on the column and in their relative activities on different cellulosic materials. Characterization of these components is in an elementary state, but the data have a bearing on the possible nature of *S* factor.

There are two ways to explain the results:

(*a*) That *Cx* and *S* factor are different—*i.e.*, that their actions are on different linkages. While the β-1,4-glucosidic linkage is widely accepted as the main link between units of the cellulose chain, the possibility of a second type of linkage has often been inferred. *S* factor could act on such a link. But it is not necessary to assume such a linkage, for action might be on the bond between carbon atoms 2 and 3 of the glucose unit, in the manner of periodate. The data seem to indicate, however, that action of *Cx* and *S* factor are on the same linkage, since under no condition have we been able to obtain a fraction having only one type of activity. The similarity of the inhibition by cellobiose and by methocel supports the view that both activities have much in common. On the other hand, the presence of a pH optimum for *S* factor in a range where *Cx* is inactive (*i.e.*, pH 3.0) indicates the possibility that the two factors may be quite different.

(b) That *S* factor is a type of *Cx*—*i.e.*, action by both is on the same linkage. The development of data indicating several hydrolytic factors (*Cx*) in filtrates leads us to the necessity for determining how each factor acts. Jermyn was able to show *Cx*'s differing in their relative activities on cellobiose and on *CMC*. Our data indicate *Cx's* differing in their relative activities on *CMC* and on swelling. As to how two enzymes acting on the same linkage can show such differences, we might suggest: (*1*) similarity to the amylases in random *vs.* endwise degradation. A high *SF/Cx* ratio might be due to a random type of action, while the reverse (low ratio) might be due to endwise attack, in which free ends would be relatively scarce in the primary wall; or (*2*) comparison of oligosaccharases with polysaccharases, in which the determining factor is chain length. Here the data of Jermyn are understandable on the grounds that a factor having a high *CB/Cx* ratio has a preference for short chains, while a factor having a low *CB/Cx* ratio has a preference for longer chains. On

this scale, our *Cx*'s, having no detectable cellobiase activity, would indicate preference for the longest chains.

The work of Jermyn [2] on the cellulolytic system of *Aspergillus oryzae* complements our own. The organism he used is unable to grow on native cellulose (cotton), and thus differs from the strongly cellulolytic organisms which form the basis of our work. Both he and we have independently concluded that there is more than one *Cx* produced by an organism; and in this we disagree with Whitaker [14]. The *Cx* components of *Aspergillus oryzae* have an action on simple β-glucosides, differing in this respect from the *Cx* components of the strongly cellulolytic organisms. On this basis, Jermyn would abolish the traditional distinction between simple β-1,4-glucosidases and β-1,4-polyglucosidases. The scheme evolving appears to be that of a family of β-glucosidases having the ability to attack substrates within a particular range of chain lengths. Whether one wishes to call these enzymes *Cx*'s or β-glucosidases (in the sense of Jermyn) is relatively unimportant. It is interesting that multiplicity of components has been demonstrated at both ends of the spectrum (*i.e.*, cellobiase/*Cx* ratio and *SF*/*Cx* ratio). The middle zone must remain undetermined until further characterization of substrate is achieved.

It should be clear from the above that, at present, we prefer to consider *S* factor a type of *Cx* in which the swelling activity is high in comparison with the action on *CMC*. This would appear to receive support from the cellobiose and methocel inhibition data, and from our inability to entirely separate the two types of activity. On the other hand, the *S* factor may possibly act on some linkage in cellulose other than the β-1,4-linkage. If it does, then *S* factor may be the enzyme C_1 which previously has been postulated.

Summary

There is an enzyme (*S* factor) present in the cellulolytic filtrates of fungi which has the ability to act on the primary wall of cotton. When placed in 18% NaOH, enzyme-treated fiber swells much more than does untreated fiber. The action appears to be on the cellulosic component of the primary wall.

Several hydrolytic enzymes (*Cx*) appear to be present in cellulolytic filtrates. The relationship of *S* factor to *Cx* varies with the organism, with growth conditions, and with conditions of purification. As a result, *S* factor may be a component entirely different from *Cx*, or it may represent an action of a particular *Cx* type. Current data favor the latter view.

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